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Pepsinogen-Like Immunoreactivity in Ascidian Stomach and Intestine: Immunohistochemical and Biochemical Study

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ABSTRACT—The reactivity of epithelial cells of digestive tract of 5 species of ascidians against antisera to an adult chicken pepsinogen (ACPg) and to an embryonic chicken pepsinogen (ECPg) was examined by indirect immunofluorescence. Stomach and intestinal epithelial cells were reactive to anti-ECPg antiserum. The localization of reactive cells in the stomach coincided with that of chief cell population which was alkaline phosphatase-positive. The protease acitivity of ascidian stomach was so weak that we could not conceive that immunoreactive substances possess peptic activity. From the results, it was suggested that the substances fulfill a function different from that of digestive enzyme, and in the course of evolution to vertebrates, the substances acquired the active site in the molecule and came to work as digestive enzyme.

INTRODUCTION

Pepsinogens are the zymogens of pepsins, digestive enzymes produced and secreted by stomach epithelial cells of vertebrates [1]. The molecular characteristics of pepsinogens and their genes have been well studied in mammals and birds, and the evolutionary course of pepsinogen genes has been suggested [2, 3]. However the origin and evolution of pepsinogns in lower vertebrates have scarcely been studied. In previous papers [4, 5], we reported the existence of pepsinogens immunologically related to an adult-type chicken pepsinogen (ACPg) in stomach gland cells of all adult vertebrate speceis examined, and of pepsinogens related to an embryonic chicken pepsinogen (ECPg) which is a prochymosin-type pepsinogen [3] in those of adult teleosts and elasmobranchs. These observations suggest that the ACPg-type and ECPg-type pepsinogens appeared early in the history of vertebrates, and in primitive groups such as teleosts and elasmobranchs these pepsinogens are co-expressed in the adult stomach, but in higher vertebrates the ACPg-type pepsinogen is predominant in adult stomach, and ECPg-type pepsinogen is expressed only during embryonic period.

We thought it interesting to seek the origin of these vertebrate pepsinogens in protochordates, the direct ancestral form of vertebrates. In this report, the presence of immunoreactive substances in the stomach and intestinal epithelial cells of adult ascidians was demonstrated. The localization pattern of immunoreactive substances in the stomach coincided with the compartmentation of epithelial cells [6, 7] and with the distribution of alkaline phosphatase activity. The stomach extract of ascidian *Styela plicata* possessed no or very low protease acitivity. Based on these facts, possible function of immunoreactive substances and their relations to the vertebrate pepsinogens will be discussed.

MATERIALS AND METHODS

Materials

Adult ascidians, *Styela plicata, Styela clava, Holocynthia hilgendorfi, Ciona savignyi,* and *Ascidia zara,* were collected at the Misaki Marine

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Methods

Indirect immunofluorescence [8] was carried out as described [4] with tissues fixed in ice-cold 95% ethanol. The antisera used were polyclonal antiserum against purified adult chicken pepsinogen (anti-ACPg) [9] and against purified embryonic chicken pepsinogen (anti-ECPg) [10], both raised in the rabbits. That these antisera are specific to each antigen was confirmed by immunofluorescence and immunoblotting [11]. Protease activity of crude extract of *Styela plicata* stomach against bovine hemoglobin was assayed by the method of Anson [12], with pH range of 2.0 to 8.0. Protease activity after electrophoresis on the polyacrylamide gel without SDS (zymogram) was visualized by the method of Samloff and Townes [13], and immunoblotting was carried out by the method described [14]. Histochemistry for alkaline phosphatase, acid phosphatase and nonspecific esterase was done according to the method described [15].

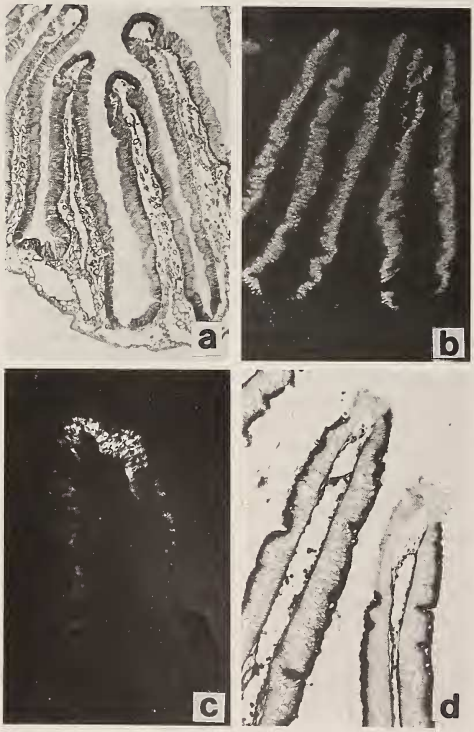

Fig. 1. Stomach of *Styela plicata*. (a) Normal histology (PAS-HX, ×63). (b) Indirect immunofluorescence with anti-ECPg. Note the absence of fluorescence at crypt and villus-top (×63). (c) Indirect immunofluorescence with anti-ACPg. Only villus-top cells are reactive (×250). (d) Alkaline phosphatase histochemistry. Villus-top cells are negative (×125).

RESULTS

Immunocytochemical study

Digestive tract of five ascidian species was examined by indirect immunofluorescence with anti-ACPg- and anti-ECPg-antisera. Results are shown in Figures. 1 to 3 and Table 1.

In all species examined, the reactivity to anti-

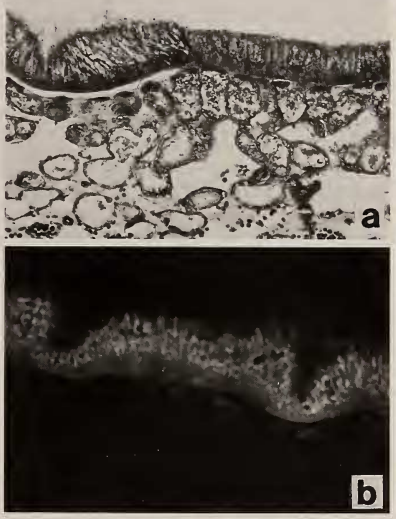

FIG. 2. Normal histology (a) and indirect immunofluorescence with anti-ECPg (b) of *Styela plicata* intestine (×175).

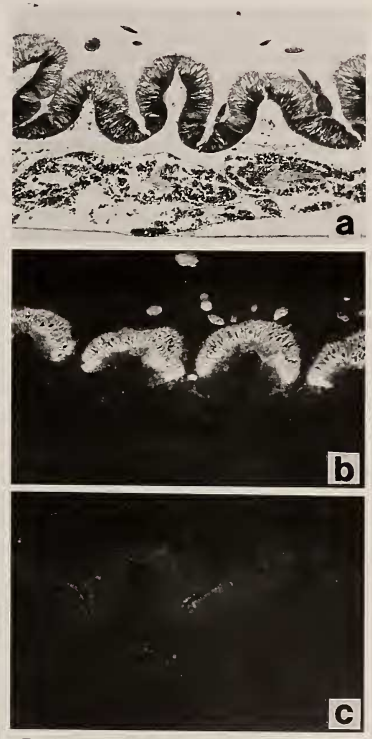

FIG. 3. Stomach of *Ciona savignyi* (×125). (a) Normal histology. (b) Indirect immunofluorescence with anti-ECPg. Cryptic part is negative. (c) Indirect immunofluorescence with anti-ACPg.

TABLE 1. Reactivity of epithelial cells of stomach villi of ascidians to anti-ECPg and anti-ACPg

Order	Genus and species	anti-ECPg			anti-ACPg		
		crypt	middle	top	crypt	middle	top
Pleurogona	*Styela plicata*	−	+	−	−	−	+
	Styela clava	−	+	−	−	−	±
	Holocynthia hilgendorfi	−	+	−	−	−	±
Enterogona	*Ciona savignyi*	−	+	+	−	−	−
	Ascidia zara	−	+	+	−	−	−

+ positive, ± weak or occasional, − negative

ECPg was found in stomach and intestinal epithelial cells. In the stomach, the localization of anti-ECPg-reactive cells differed between Pleurogona species and Enterogona species (Table 1). In the former, the stomach has long villi (Fig. 1a) and epithelial cells intermediate between villus-top and crypt were reactive to the anti-ECPg antiserum (Fig. 1b), while in the latter all epithelial cells of short villi (Fig. 3a) except those of crypt were reactive (Fig. 3b). Intestinal epithelial cells were always reactive to the anti-ECPg antiserum (Fig. 2a, b). In the digestive organs other than the stomach and intestine, only small number of gland cells of endostyle showed very weak fluorescence when they were treated with anti-ECPg.

Villus-top cells of the stomach of Pleurogona species were often weakly reactive to anti-ACPg (Fig. 1c) while stomach epithelial cells of Enterogona species showed virtually no reactivity against anti-ACPg (Fig. 3c).

Alkaline phosphatase histochemistry

To examine the possible functional significance of cells immunoreactive to anti-ECPg in ascidian digestive tract, we studied alkaline phosphatase activity of the stomach and intestine of *Styela plicata.* Alkaline phosphatase activity was demonstrated on apical part of epithelial cells of the somach (Fig. 1d) and intestine, and the localization of alkaline phosphatase-positive cells coincided with that of anti-ECPg-positive cells, the cells of cryptic part and villus-top being negative to alkaline phosphatase. Acid phosphatase and nonspecific esterase were not detected in the stomach or intestine.

Protease activity in crude extract of Styela plicata *stomach*

Next, we examined whether anti-ECPg-reactive substances have protease activity or not. We measured protease activity in crude extract of the stomach at various pHs against hemoglobin or albumin as substrate. The results are summarized in Table 2, in which protease activity of smooth dogfish is also cited as a reference. From the Table it is obvious that protease activity of *Styela* stomach is very low compared to that of dogfish; it is about 1/500 of that of dogfish. There is no pH-dependency and substrate-dependency. When the activity was measured at 15°C or 20°C, no increase in activity was observed, and the activity was not inhibited by pepstatin, a specific inhibitor of acid proteases (data not shown). On the zymogram made with 10% polyacrylamide gel without SDS, we could not detect the band of activity (Fig. 4) nor the immunoreactive band after immunoblotting (data not shown). We also measured the activity in whole homogenate or supernatant after extraction with several kinds of detergent, such as SDS, Tween 20 or NP40, but the results were

Table 2. Protease activity in stomach extract of *Styela plicata* and smooth dogfish at 37°C

Animal	Substrate	pH	Specific activity (units/mg protein)
S. plicate	Hemoglobin	2.0	0.010
		3.0	0.018
		4.0	0.003
		5.0	0.004
		6.0	0.001
		7.0	0.005
		8.0	0
	Albumin	3.0	0.002
		7.0	0.002
Smooth dogfish	Hemoglobin	2.0	9.6
		8.0	0

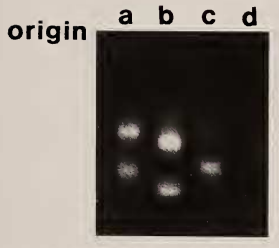

Fig. 4. Zymogram for acid protease (pH 2.0) in crude extract of the stomach taken from mouse (lane a), chicken (lane b), smooth dogfish (lane c) and *Styela plicata* (lane d). After electrophoresis, gel was immersed in hemoglobin solution (pH 2.0) and then incubated at 37°C for 1 hr. Hemoglobin was stained with amidoblack. The sites of acid protease activity appear as white bands.

always the same as in the case of extraction without detergent (data not shown).

DISCUSSION

It is well known that vertebrate stomachs have pepsinogens as proenzymes for digestion under acidic conditions [1]. We have demonstrated that vertebrate stomachs produce at least one pepsinogen species which is immunologically related [4]. This pepsinogen is called "adult-type" since it is detectable by the antiserum raised against ACPg [9]. Moreover, stomach epithelial cells of adult teleosts and elasmobranchs have pepsinogen immunoreactive to the antiserum against ECPg [5].

In the present study, we found that anti-ECPg-reactive substances exist in epithelial cells of the stomach and intestine of all ascidian species examined. Their localization in the stomach is interesting from the point of view of function and compartmentation of epithelial cells. The compartments of epithelial cells in ascidian stomach were studied by Ermak [6, 7], by autoradiography with tritiated thymidine. The author revealed that there exist two types in stomach epithelial cells; chief cell population and mucous cell populations. Each population has its germinal zone. In Phleobranchs such as *Ciona,* mucous cell population is confined in one part of stomach, but in Styelids mucous cell populations exist on each villus-top. Our immunocytochemical observations clearly demonstrated that cells in chief cell population except its germinal zone are reactive to anti-ECPg (Fig. 1b). These cells possess also alkaline phosphatase activity. These results indicate that chief cell population is different from mucous cell population not only in cell kinetic properties but also functionally.

Although it is presumed that anti-ECPg-reactive substances in chief cell population have some functional significance, we detected only very low protease activity in stomach extract. As for the protease activity of ascidian stomach, Ikeda *et al.* [16] reported considerable activity in *Microcosmus* stomach at pH 3.6 against hemoglobin. However, Giraud and Yeomans [17] stated that protochordate (*Polycarpa*) stomach does not contain measurable amount of pepsinogen. We measured the protease activity against hemoglobin or albumin at wide range of pH (2.0–8.0) but the values of specific activity were 1/500 to 1/5000 of those of vertebrate stomachs. It may be nevertheless possible that anti-ECPg-reactive substances in ascidians are acid proteases, and that their proteolytic nature is different from that of vertebrate acid proteases. But we have at present no convincing data to support the idea that the pepsinogen-like substances in ascidians play definite role in ascidian digestion.

Thus the function of anti-ECPg-reactive substances is not clear at present, but we can imagine as a tentative hypothesis possible relation of the substances and vertebrate ECPg-like pepsinogens as follows: The anti-ECPg-reactive substances in ascidians fulfill a non-digestive function, and in the evolutionary course to vertebrates, these substances came to possess protease activity presumably by acquiring the active site in the molecules. To test this hypothesis, we have to clone the gene for ECPg-like molecule in ascidians and compare its structure with ECPg gene which has already been cloned [18], with special attention to the base sequence of active center that is well conserved in vertebrate pepsinogens. In this regards, our cDNA probe of ECPg [3] will be helpful.

The presence of anti-ACPg-reactive substance in mucous cell population of *Styela plicata* is very

curious, and it deserves further studies. In adult teleosts and elasmobranchs, single cells seemed to produce ECPg- and ACPg-like pepsinogens at the same time [5]. The seggregation of cell types reactive to two antisera in the ascidian stomach raised a possibility that ECPg-like and ACPg-like molecules were produced in different cell population in vertebrate ancestor. This problem, as well as the fact that anti-ECPg-reactive substances exist also in intestinal epithelial cells of ascidians, is very important in elucidating the origin and evolution of stomachs in vertebrates.

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